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Choline accumulation by photoreceptor cells of the rabbit retina

(transport/phospholipids/membranes/synthesis)

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ABSTRACT Photoreceptor cells of the rabbit retina accumulate choline from the extracellular environment by an overall process that has a high affinity for choline. These cells do not synthesize acetylcholine; instead, the choline taken up is incorporated into phosphorylcholine and eventually phospholipid. A mechanism for efficient choline accumulation is presumably concomitant to the photoreceptor cell's synthesis of large amounts of membrane for outer segment membrane renewal. Its existence in the photoreceptor cell supports previous evidence that high-affinity choline uptake is not confined to neurons that release acetylcholine, but may be present wherever large amounts of choline are required.

The light-sensitive element of mammalian retinas is the outer segment of the photoreceptor cell. In the rod type, the better-understood and generally more frequent, the outer segment may be visualized as a thin cylinder enclosing a stack of membrane discs. The discs are densely packed (a rat rod contains about 900), so that the amount of rhodopsin-containing membrane possessed by each photoreceptor cell is large. The tips of the outer segments are shed, in a cyclic daily rhythm, and phagocytosed by the neighboring pigment epithelium (1–7). In order to maintain a steady state, then, each photoreceptor cell must replace the discarded membrane, and the amount of new membrane that must be provided is very great. From the rate of renewal of outer segments (5) and their dimensions one may calculate that each rat rod replaces between 200 and 600 μm^2 of membrane per day. This is probably one of the higher rates of membrane synthesis anywhere in the body—and is surely extreme for members of a class of cell that ceases dividing shortly after the animal's birth.

Our interest in this process originated in the course of studies aimed at the autoradiographic identification of a class of retinal cell known from other evidence to synthesize acetylcholine. We exposed isolated rabbit retinas to 0.1–0.3 μM [^3H]choline, on the assumption that low concentrations would selectively favor choline's accumulation by the high-affinity process observed in neurons that synthesize acetylcholine (8–10). The retinas did synthesize [^3H]acetylcholine, but they synthesized even more [^3H]phosphorylcholine, which was later incorporated into phospholipids, primarily phosphatidylcholine (11). Phosphatidylcholine comprises about 45% of the total phospholipid found in outer segments (12, 13).

The same study provided autoradiographic evidence that the rabbit retina's acetylcholine synthesis occurs in a sparse group of cells that line both margins of the inner plexiform layer (ref. 11; see also ref. 14). The majority of the retina's total synthesis of choline-containing phospholipids, but essentially none of its acetylcholine synthesis, occurs in the photoreceptors (11). This was in accord with the finding of Ross and McDougal (15) that rabbit photoreceptors contain a negligible amount of choline acetyltransferase.

Taken together, these observations suggested that the photoreceptors might accumulate choline by a high-affinity mechanism, but use the choline not for the formation of acetylcholine but for the eventual synthesis of phospholipid. We have therefore carried out a conventional kinetic analysis of the retina's overall choline uptake and have studied the choline metabolites formed at each choline concentration. A considerable fraction of the retina's phosphorylcholine synthesis was found to be limited by a saturable process with a high affinity for choline. That this occurred primarily in the photoreceptors was confirmed by measurement of the choline uptake that had taken place in photoreceptor cells microdissected from freeze-dried retinas.

MATERIALS AND METHODS

Rabbit retinas were quickly removed from the pigment epithelium and incubated at 37°C and pH 7.4 in 6-ml rocking tubes (16) containing (mM): Na^+, 143.0; K^+, 3.6; Ca^{2+}, 1.15; Mg^{2+}, 1.2; Cl^-, 125.4, HCO_3^-, 22.6; $H_2PO_4^-$, 0.1, HPO_4^{2-}, 0.4; SO_4^{2-}, 1.2; glucose, 10. The medium was equilibrated with 5% CO_2/95% O_2. Extensive precautions to prevent the contamination of the medium by possibly toxic substances were taken. Previous experiments have shown that rabbit retinas incubated in this way maintain their protein synthesis (17), morphological integrity (18), photoreceptor sensitivity (19), electrolyte balance (20), and electrophysiological function as judged by ganglion cell receptive fields (21) for at least 6 hr. Most of the experiments described here were carried out on retinas from New Zealand white rabbits; as a control for eye pigmentation a few retinas from New Zealand red rabbits were also studied, with similar results.

After a 10-min preincubation in medium containing no choline the retinas were transferred for 5 min to medium containing [*methyl*-^3H]choline (New England Nuclear) at concentrations from 0.4 to 300 μM. Specific activities ranged from 84 to 0.39 Ci/mmol, depending on the amount of unlabeled choline added to achieve the final concentrations (1 Ci = 3.7×10^{10} becquerels). At the end of the labeling period the retinas were removed, rinsed for 1 min in unlabeled medium, and cut into symmetrical halves. One half-retina was touched three times to glass (to remove adhering medium) and dropped into a tared glass homogenizing tube containing 1 ml of formate/acetate buffer (pH 1.9) and 0.1 mM physostigmine. The tube was quickly weighed and the retina was homogenized. Aliquots of the homogenate were counted in Aquasol (New England Nuclear); internal toluene standards were used. The remaining homogenate was centrifuged at 3000 × g, and the supernatant was analyzed for radioactive acetylcholine, phosphorylcholine, and choline by high-voltage paper electrophoresis (22–24). Negligible quantities of radioactive phospholipid or betaine were present in retinas labeled for 5 min.

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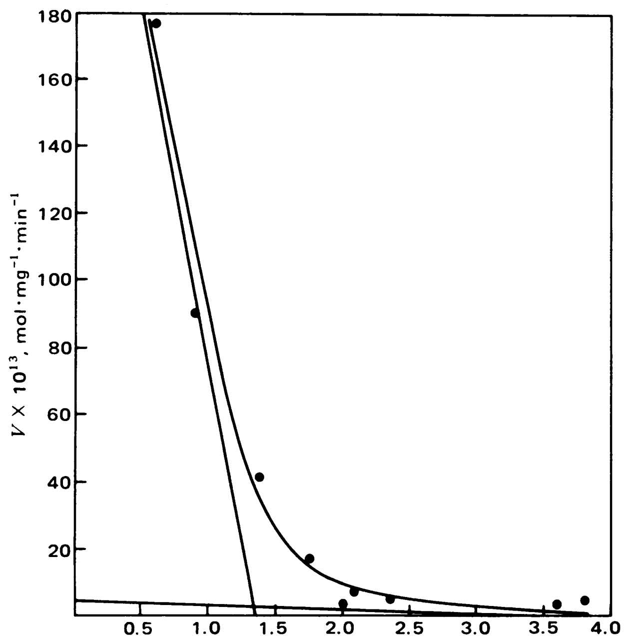

FIG. 1. Hofstee plot of the intact retina's choline uptake. The overall velocity of uptake (V) is plotted as a function of the ratio of the velocity to the concentration of choline (V/S). The data have been analyzed as the sum of two Michaelis–Menten components, which are represented by straight lines. The high-affinity component had a K_m of 1.4 μM and a V_{max} of 0.42 pmol $\times$ mg^{-1} $\times$ min^{-1}. The low-affinity component had a K_m of 208 μM and a V_{max} of 28.4 pmol $\times$ mg^{-1} $\times$ min^{-1}. The curved line represents the sum of the two processes calculated from these parameters. Each point shows the mean for four retinas. SEMs of experimental V in this and all other figures were less than 17%.

Small pieces from the other half-retina were plunged into a propane slurry at liquid nitrogen temperature, slowly freeze-dried under reduced pressure, lightly fixed with osmium vapor, and vacuum-embedded in Spurr's resin (25). The photoreceptors were isolated by microdissection from 10-μm vertical sections. The sections were laid flat on the stage of a high-power (×80) dissecting microscope. A cut parallel to the outer plexiform layer and passing through the lower third of the outer nuclear layer was made with a razor blade (see Fig. 4). The isolated photoreceptors were photographed and the accuracy of the dissection was verified on a large print. Their image was cut out and weighed. The volume of tissue was established from the surface area thus measured and the section's known thickness. The sections containing the microdissected photoreceptors were immersed for 45–50 hr in formate/acetate buffer, and the radioactivity extracted into it was established by scintillation counting. Previous work has shown that this buffer penetrates Spurr's resin thoroughly, so that more than 85% of the water-soluble choline metabolites are extracted. The radioactive choline compounds are stable under the conditions to which they are exposed during processing, and the choline metabolites extracted from sections faithfully reflect those present in the tissue at the end of incubation (11). Because it allows identification of acetylcholine in the extract, we used the acid extraction for most of the studies presented here; results of the kinetic studies, in which the total uptake of radioactivity was the only concern, were verified in a smaller series in which the isolated photoreceptors were completely solubilized by strong base ("NCS", Amersham). These techniques have been described in detail in earlier publications (11, 21, 24).

Kinetic analysis was performed by conventional graphic methods by using Hofstee plots. The observed velocity of choline accumulation was plotted as a function of the ratio of the velocity to the choline concentration, and the experimental values were analyzed as the sum of two Michaelis–Menten components. Kinetic parameters were established by using a nonlinear least-squares computer program written by W. W. Cleland (26, 27) and adapted to the DEC-10 computer by Baughman and Bader (14). In this analysis we have followed the conventional practice of ignoring any outward choline flux

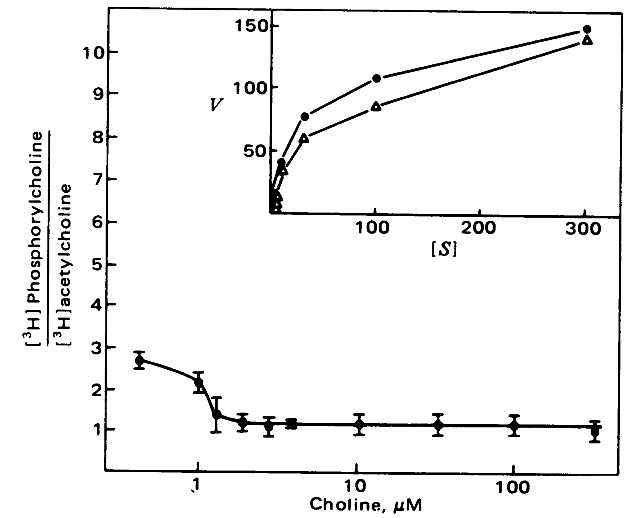

FIG. 2. Relative rates of accumulation of [^{3}H]phosphorylcholine and [^{3}H]acetylcholine at increasing choline concentrations. (*Inset*) Velocities (10^{14} $\times$ mg^{-1} $\times$ min^{-1}) from which the ratios were derived. Note that the rate of synthesis of [^{3}H]phosphorylcholine (●) and of [^{3}H]acetycholine (△) increase in an overall manner that is very similar, and that phosphorylcholine synthesis is favored at the lowest concentrations—those below saturation for the high-affinity process shown in Fig. 1.

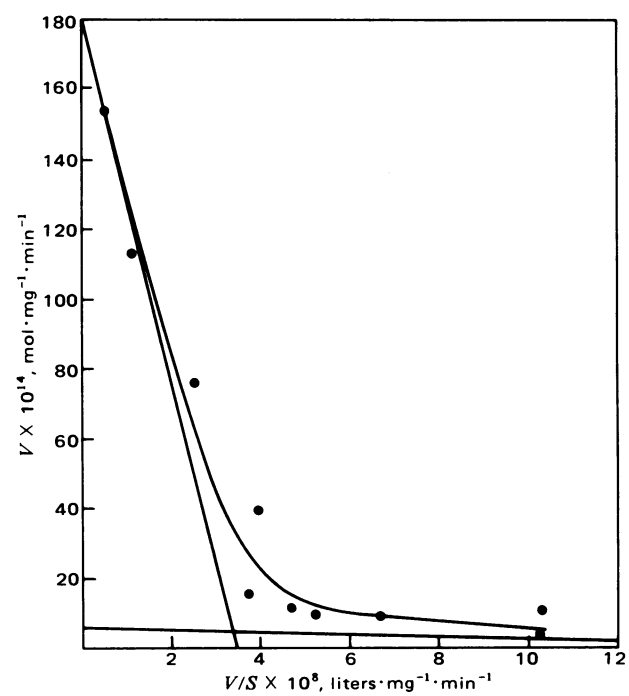

FIG. 3. Hofstee plot of rates at which [^{3}H]phosphorylcholine appeared in the retinas. Conventions as in Fig. 1. The observed velocities have been represented as the sum of two saturable processes, one with a K_m of 0.4 μM and a V_{max} of 0.056 pmol $\times$ mg^{-1} $\times$ min^{-1} and the other with a K_m of 50 μM and a V_{max} of 1.73 pmol $\times$ mg^{-1} $\times$ min^{-1}.

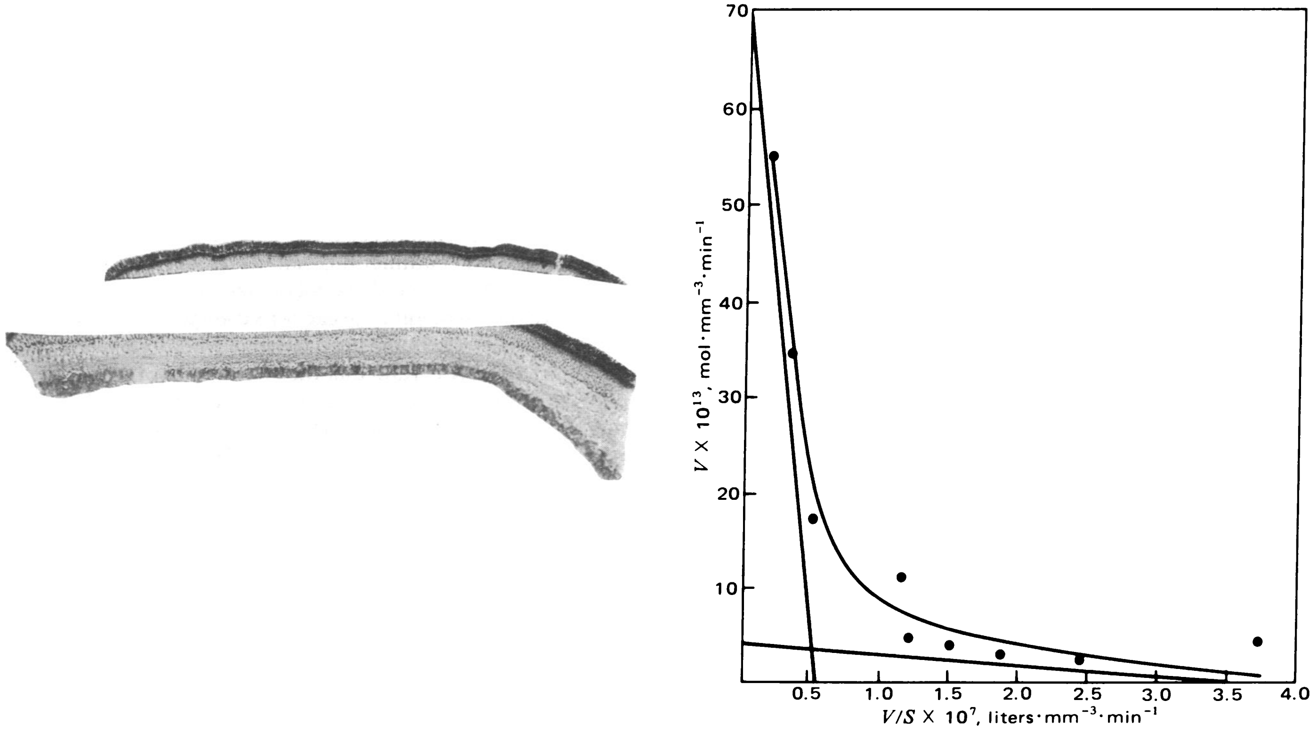

FIG. 4. Isolation of photoreceptor cells and analysis of their radioactive contents. (*Left*) Micrograph shows a 10-μm-thick section. A cut has been made parallel to the outer plexiform layer. The cut passes through the lower part of the outer nuclear layer, isolating some of the photoreceptor cells from the rest of the retina. The isolated photoreceptors were teased apart from the rest of the section for analysis. (×90.) Radioactivity contained in photoreceptor cells isolated as shown was extracted into acid and referred to the volume of tissue established from photographs of the dissected sections. (Similar results were obtained in a smaller series of sections completely solubilized by NCS.) Kinetic analysis as described in Fig. 1 was carried out (*Right*). The accumulation of choline within the photoreceptor cells could be represented by two Michaelis–Menten components. The high-affinity component had a K_m of 1.4 μM and a V_{max} of 0.5 pmol × mm^{-3} × min^{-1}. The low-affinity component had a K_m of 128 μM and a V_{max} of 7 pmol × mm^{-3} × min^{-1}. The tissue samples analyzed in this way were pieces cut at the end of incubation from the retinas whose total uptake is shown in Fig. 1. Each data point shows the mean for 18 extracted sections: three sections per block for each of three blocks for two retinas.

that might have occurred during the measurement (17). For this reason, we have preferred to speak of the data presented in Figs. 1, 3, and 4 as describing the overall kinetics of choline accumulation, an operational phrase closely linked to the experimental manipulations. For present purposes the distinction is minor, because the primary concern was only to learn how the photoreceptor cells might differ from others in their effectiveness at capturing extracellular choline.

RESULTS

As expected from studies in the chicken (14), turtle (28), and rat (29), the retina accumulated choline in two kinetically separable phases, one of which was characterized by a high overall affinity for choline (Fig. 1).

Table 1. Effect of hemicholinium-3 and low-Na^+ medium on the retina's total uptake of choline and synthesis of choline metabolites

	pmol × 10^2 · mg^{-1} · min^{-1}		
	Total radioactivity	[^{3}H]Acetyl-choline	[^{3}H]Phosphoryl-choline
Control	10.41 ± 1.07	1.40 ± 0.25	3.81 ± 0.50
23 mM Na^+	7.29 ± 0.80	0.25 ± 0.04	2.50 ± 0.24
Hemicholinium-3	3.05 ± 0.28	0.10 ± 0.01	4.68 ± 0.76

The concentrations of choline and hemicholinium-3 were selected from published data (10, 30, 31) to provide a maximally selective effect on high-affinity choline uptake. At higher concentrations of hemicholinium-3, high-affinity and low-affinity uptake are blocked, and the synthesis of both choline metabolites by the retina is depressed (11). Data are expressed as mean ± SEM for at least four retinas per condition.

The first indication that the photoreceptors might accumulate choline by the high-affinity process came from comparison of the rates of synthesis of [^{3}H]phosphorylcholine at varying [^{3}H]choline concentrations (Fig. 2). At all concentrations, more [^{3}H]phosphorylcholine than [^{3}H]acetylcholine was formed. Furthermore, the relative amounts of the two metabolites remained constant over most of the range, maintaining a ratio of about 1.2 to 1. If the synthesis of [^{3}H]phosphorylcholine and [^{3}H]acetylcholine were limited by two kinetically different processes, their rates of synthesis should have diverged as the velocities of the two processes diverged. Instead, if there was a change in the relative amounts of [^{3}H]phosphorylcholine and [^{3}H]acetylcholine, it was in the unexpected direction: relatively more phosphorylcholine was synthesized at the lowest choline concentrations, at which (from Fig. 1) the relative velocity of the high-affinity process was greatest. We therefore studied the rates at which [^{3}H]phosphorylcholine was formed and again found both high- and low-affinity components (Fig. 3). At low concentrations of choline, then, retinal phosphorylcholine synthesis appears to be limited by a saturable process with K_m in the order of 1 μM.

Under some circumstances one might seek to explain these findings by postulating that most of the observed [^{3}H]phosphorylcholine was formed within acetylcholine-synthesizing neurons, and thus used choline that became available via those cells' high-affinity uptake system. But in the retina this cannot be the case. The cholinergic cells make up less than 1% of the retina's neurons (11, 14) and synthesize a negligible fraction of the retina's total phospholipid (11); the majority of the retina's phosphorylcholine synthesis occurs in the photoreceptors. The findings just described thus suggested very strongly that the photoreceptors have a high-affinity choline uptake that leads to phosphorylcholine synthesis. We sought additional evidence by directly measuring the radioactivity taken up by the photoreceptors.

Small pieces from the retinas whose radiochemical contents are shown in Figs. 1–3 were quickly frozen at the end of incubation, freeze-dried, and embedded under reduced pressure. Ten-micrometer sections were divided under ×80 magnification along a line running near the inner edge of the outer nuclear layer. The accuracy of the cut and the volume of photoreceptor tissue were established for each section from large photographic prints. The radioactivity contained in the microdissected section was extracted into acid and counted. Kinetic analysis unequivocally showed that choline had accumulated in the photoreceptor cells by a process characterized by a high affinity for choline (Fig. 4).

An additional series of retinas was incubated in the presence of 0.4 μM extracellular choline, but in the presence of 1 μM hemicholinium-3 or in medium in which Na^+ was decreased to 23 mM by substitution of LiCl for NaCl. The retinas were homogenized and their radiochemical contents were identified by electrophoresis. Both hemicholinium-3 and low-sodium medium caused a major decrease in the amount of [^{3}H]acetylcholine synthesized, but the only effect on the synthesis of [^{3}H]phosphorylcholine was a 34% decrease in the low-sodium medium (Table 1).

DISCUSSION

We have presented two independent kinds of evidence that the photoreceptor cells of the rabbit retina possess high-affinity choline uptake. The first comes from studies of the whole retina, in which it was found that the synthesis of radioactive phosphorylcholine from exogenous [^{3}H]choline (which is dominated by the photoreceptors) is limited at low choline concentrations by a process characterized by a high overall affinity for choline. The second comes from direct kinetic analysis of choline uptake in photoreceptor cells isolated from freeze-dried tissue. Each of these two experiments would be rather persuasive by itself; together they seem quite conclusive.

They indicate that high-affinity choline uptake, kinetically defined, is not exclusively a property of cholinergic neurons. There is substantial precedent for such a conclusion from studies in which tissue cultures of glial cells (32), fibroblasts (33), neuroblastoma cells (34, 35), embryonic cortex (36), and hepatoma cells (37) have all been shown to have high-affinity choline uptake without acetylcholine synthesis. Two explanations have been offered for the presence of high-affinity uptake in these cells. One is that the cells grown under isolated conditions are expressing a gene (for the high-affinity carrier) that they would not normally express—that they are synthesizing an unnecessary carrier. The second is that the cultured cells are growing rapidly compared to most neurons and might have high-affinity choline uptake as one of the mechanisms that allows rapid synthesis of cell membranes.

In many of the studies of cultured cells, choline uptake was found not to be inhibited by hemicholinium-3 (34, 38) and to be incompletely inhibited by incubation media containing low concentrations of Na^+ (33, 34, 38). The accumulation of choline by acetylcholine-synthesizing neurons, in contrast, is very sensitive to these treatments (8–10). In the present experiments the synthesis of phosphorylcholine was unaffected by hemicholinium-3 and was depressed only 34% by low Na^+ medium, whereas the synthesis of acetylcholine was virtually eliminated in both cases. Thus, the overall process by which choline is taken up from the medium and incorporated into phosphorylcholine in the photoreceptors, like that in the cultured cells, is pharmacologically distinguishable from the process that leads to acetylcholine formation in other neurons. One possibility (an uneconomical one) is that a high-affinity carrier mediating the uptake of choline by cells that synthesize only phosphorylcholine is different from the one that mediates choline uptake in acetylcholine-synthesizing cells. However, one must remember that experiments of the type discussed here measure overall choline accumulation, not its transport across cell membranes.[§] The observed overall uptake must depend to some extent both on the membrane carrier's transport of choline and on the choline's subsequent acetylation or phosphorylation; and it is not yet entirely certain that the effects of hemicholinium-3 or low Na^+ medium are not on the latter processes rather than the former. Until rigorous transport measurements are made, the existence of two different high-affinity carriers should perhaps remain an open question.

These pharmacological issues do not affect our main conclusion, which is that the photoreceptor cells of the rabbit retina accumulate choline by an overall process that is very effective at low choline concentrations. This specialization presumably reflects the photoreceptors' need to synthesize choline-containing membranes for outer segment replacement. Its presence supports the suggestion that high-affinity choline uptake may be a property not only of cells that synthesize acetylcholine but also of any that have a large requirement for choline, and emphasizes the degree to which the overall biology of photoreceptor cells must reflect their continuous structural renewal.

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§ The demands of the latter measurement, which include measurement of outward as well as inward solute fluxes, have been discussed by Ames *et al.* (17, 39).

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